ISOLATION AND CHARACTERIZATION OF MARINE ACTINOMYCETES FOR ANTI-INFECTIVE ACTIVITY TOWARDS *Pseudomonas aeruginosa* PA14

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by

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LIST OF ABBREVIATIONS

µg	Microgram
µL	Microlitre
ASW	Artificial Sea Water
$BaCl_2$	Barium chloride
$CaCl_2$	Calcium chloride
$CaCO_3$	Calcium carbonate
$CHCl_3$	Chloroform
DCM	Dichloromethane
$FeSO_4.7H_2O$	Iron sulphate 7-hydrate
g/L	Gram per litre
GC-MS	Gas Chromatography Mass Spectrometry
GFP	Green Fluorescent Protein
H_2SO_4	Sulphuric acid
HCl	Hydrochloric acid
IGF-1	Insulin-like Growth Factor 1
ISP1	Tryptone-Yeast extract broth
ISP2	Yeast extract-malt extract broth
K_2HPO_4	Potassium phosphate dibasic
KCl	Potassium chloride
KNO_3	Potassium nitrate
KPO_4	Potassium phosphate
LB	Lysogeny Broth
M	Molar
MeOH	Methanol
Mg	Milligram
$MgSO_4$	Magnesium sulphate
$MgSO_4.7H_2O$	Magnesium sulphate 7-hydrate
mL	Millilitre
Mm	Millimetre

mM	Millimolar
N	Normality
NA	Nutrient Agar
Na_2HPO_4	Sodium phosphate dibasic
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NIST	National Institute of Standards and Technology
Nm	Nanometer
OD	Optical density
Rcf	Relative centrifugal force
R_f	Retention factor
Rpm	Revolution per minute
SD	Standard deviation
sp.	Species
TD50	Median toxic dose
TLC	Thin Layer Chromatography
v/v	Volume per volume
w/v	Weight per volume

PEMENCILAN DAN PENCIRIAN AKTINOMISET MARIN UNTUK AKTIVITI ANTI-JANGKITAN PADA *Pseudomonas aeruginosa* PA14

ABSTRAK

Persekitaran marin berfungsi sebagai takungan luas metabolit sekunder berguna dan pada masa yang sama, agen anti-jangkitan baru dikehendaki untuk memerangi bakteria patogen tahan antibiotik. Kajian ini dijalankan untuk menyaring agen anti-jangkitan daripada persekitaran yang mampu mengatasi jangkitan *Pseudomonas aeruginosa* melalui anti-virulen atau meningkatkan aktiviti imuniti perumah. Mendapan tanah laut dipungut dari Pulau Songsong, Yan, Kedah, Malaysia dan sebanyak 18 aktinomiset dengan morfologi yang berbeza telah berjaya dipencilkan. Metabolit sekunder dari semua pencilan telah diekstrak dengan menggunakan kaedah pengekstrakan pepejal-cecair dan telah disaring untuk aktiviti anti-jangkitan menggunakan model jangkitan *Caenorhabditis elegans*. Hanya ekstrak dari pencilan A5 menunjukkan pertambahan jangka hayat yang ketara daripada perumah. Oleh itu, ia telah digunakan untuk pencirian dan saringan lanjut. Pencilan A5 menunjukkan 99.7% persamaan jujukan molekular kepada *Streptomyces sundarbansensis* MS1/7 dan telah dinamakan sebagai *Streptomyces sundarbansensis* CCB-PSK207 kerana perbezaan morfologi yang diperhatikan di bawah kondisi percambahan yang sama. Ia boleh menahan sehingga 10% (w / v) kepekatan NaCl dan menunjukkan pertumbuhan tertinggi (merujuk kepada berat kering sel) pada pH7 selepas sepuluh hari percambahan berbanding dengan pH4, pH5, pH6, pH8, pH 9 dan pH10. Ekstrak metanol A5 seterusnya dikelaskan lagi menggunakan kaedah pengekstrakan cecair-cecair. Pecahan

n-heksana didapati mempamerkan hit yang terbaik dalam asai ketahanan. Asai tindak balas dos mendedahkan bahawa 400μg/mL adalah kepekatan yang terbaik dengan perubahan kadar ketahanan 69.65±4.50% berbanding cacing kawalan selepas 96 jam jangkitan PA14. Oleh itu, semua asai penyaringan telah diteruskan dengan kepekatan tersebut. Pecahan ini tidak menunjukkan kesan ke atas pertumbuhan kinetik PA14 dan ia juga tidak menyebabkan sekatan pemakanan dalam perumah. Dalam asai visualisasi *lys-7*, ekspresi GFP yang signifikan telah diperhatikan dalam cacing jangkitan PA14 yang menerima 400 μg/mL pecahan *n*-heksana. Intensiti GFP yang kuat telah dipamerkan di seluruh badan cacing yang mencadangkan bahawa ekspresi gen *lys-7* dalam populasi cacing yang dijangkiti telah dipulihkan. Pecahan ini seterusnya diasingkan oleh kaedah kromatografi lapis tipis preparatif. Tiga fraksi iaitu (A5HA, A5HB dan A5HC) telah diasingkan dan sekali lagi disaring untuk asai ketahanan. Fraksi A5HB menunjukkan hit yang terbaik dengan perubahan 71.43±4.67% kadar ketahanan berbanding cacing yang tidak dirawat. Sebatian fraksi telah dikenal pasti oleh analisis GC-MS dengan kehadiran beberapa metil ester asid lemak tepu dan bercabang. Kesimpulannya, fraksi mengandungi sebatian kompaun dengan keupayaan menyelamatkan *C. elegans* melalui aktiviti pengantara *lys-7*.

ISOLATION AND CHARACTERIZATION OF MARINE ACTINOMYCETES FOR ANTI-INFECTIVE ACTIVITY TOWARDS *Pseudomonas aeruginosa* PA14

ABSTRACT

Marine environment serve as reservoir of vast useful secondary metabolites and at the same time, new anti-infective agents are required to combat antibiotic resistant pathogenic bacteria.Therefore, this research was carried out to screen for anti-infective agents from the environment that are capable of overcoming *Pseudomonas aeruginosa* infection through anti-virulence or boosting of host immunity activities. Marine soil sediments were collected from Songsong Island, Yan, Kedah, Malaysia and a total of 18 actinomycetes with different morphology were successfully isolated. Secondary metabolites from all isolates were extracted out using solid-liquid extraction method and were screened for anti-infective activity using *Caenorhabditis elegans* infection model. Only extract from isolate A5 showed significant life-span promotion of the host. Thus, it was used for further characterization and screening. Isolate A5 showed 99.7% molecular sequence similarity to *Streptomyces sundarbansensis* MS1/7 and was designated as *Streptomyces sundarbansensis* CCB-PSK207 due to morphological difference observed under the same culture condition. It can withstand up to 10% (w/v) NaCl concentration and it showed highest growth (referring to cell dry weight) at pH7 after ten days of cultivation compared to pH4, pH5, pH6, pH8, pH 9 and pH10. Methanolic extract A5 was further partitioned by using liquid-liquid extraction method. *n*-hexane partition was found to exhibit the best hit in survival assay. Dose response assay revealed that

400µg/mL was the best concentration with survivability rate change of 69.65±4.50% as compared to controlled worms 96-hours post PA14 infection. Therefore, all screening assays were progressed using that concentration. The partition showed no effects on the kinetic growth of PA14 and it also did not cause dietary restriction in the host. In *lys-7* visualization assay, a significant GFP expression was observed in PA14-infected worm that received 400 µg/mL n-hexane partition treatment. Strong GFP intensity was exhibited all over the worm body which suggested that the expression of *lys-7* gene in the infected worm population was restored. The partition was further isolated by preparative thin layer chromatography method. Three fractions (A5HA, A5HB and A5HC) were isolated and once again screened for survival assay. Fraction A5HB showed the best hit, with 71.43±4.67% survival rate change compared to the untreated worms. Compounds in the fraction were identified by GC-MS analysis with presence of several methyl esters of saturated and branched fatty acids. In conclusion, the fraction contains compounds with capability of rescuing *C. elegans* through *lys-7* mediated activity.

CHAPTER 1

1.0 Introduction

Pseudomonas aeruginosa is listed as the multi-drug resistant Gram-negative pathogen. Since it was first observed in 1850 and until now, it is still an infection which needed research and new development for treatment opportunity (Lister *et al*., 2009). Therefore, it is very worrying that we are running out of antibiotics option. In this situation, opportunity in developing methods to control and treat infection of *P. aeruginosa* is an important step. However, the development of other drugs will cause resistance among bacteria with encoded resistance gene. Therefore, a good choice of drug is the one not only will target the bacterial virulence factor but also will modulate the host defence. The selectivity of anti-infective in which only non-essential genes are being targeted for disruption impose a lower probability of resistance development (Mellbye and Schuster, 2011) and at the same time preserve cell viability of the host.

While the search of new therapeutic drugs from nature to combat the pathogen infection is continuously being carried out, researchers found that actinomycetes are popular among antibiotics and other drug producers which might be a potential therapeutic drug for pathogen infections. Marine actinomycetes are less popular as compared to terrestrial actinomycetes but their potentials are undeniable. Recently, marine environment is becoming a spotlight for novel drugs excavation because it is under-explored but rich in diversity of bacteria as most bacteria in the sea was found to help in degradation and cycle of organic matter (Lam, 2006). Studies have proven that actinomycetes isolated from the marine environment are capable to produce novel

secondary metabolites and have adapted to life in the sea. They also have different characteristics from those of the terrestrial group because of the different environmental conditions. So, it gives a run down that they might produce different types of bioactive compounds (Imada *et al.,* 2007) making up an interest to study the marine actinomycetes as a new therapeutic method to combat *P. aeruginosa* infection.

In the development of new therapeutic drugs, most of studies will use animal or tissue culture for drug testing as they are close to human cellular and development systems. However, the use of animal for drug testing needs a lot of space, time consuming, high cost and most importantly considered against the animal ethics. On the other hand, the use of tissue culture is a tedious work, as maintaining the cells and keeping it free from contaminant is easier said than done. Therefore, application of *Caenorhabditis elegans* as host infection model is a better approach as compared to other conventional method. Nowadays, *C. elegans* has been widely used as it is very easy to be cultivated in the laboratory with a short life-cycle of about three days of cultivation (Riddle *et al.,* 1997). Moreover, it complements both *in vitro* and *in vivo* mammalian models in toxicology due to its success in genetic manipulability, invariant and fully described developmental program and well-characterized genome (Leung *et al*., 2008). Besides, *C. elegans* also have clear ortholog with human genes (Shaye and Greenwald, 2011; Kaletta and Hengartner, 2006; Culetto and Satelle, 2000). Therefore, it makes *C. elegans* a close relative to the human system and the most suitable host for toxicology and drug testing upon all of its characters.

1.1 Problem statements

There is abundance of infectious cases reported to be related to *P. aeruginosa.* Antibiotics have been invented but drug resistance appear to be another problem when dealing with living organisms. This is the biggest problem faced by the global community that lead to this research. Therefore, this research was meant to look for another initiative to treat the infection by stimulating immunity of the host rather than killing the pathogen.

Many compounds have been discovered with abilities as anti-infective agents that were isolated from plants and even microorganisms. But only terrestrial microorganisms were mostly studied for that intention while marine microorganisms are being underexplored. Not much are known about the capabilities of marine microorganisms in producing useful therapeutic agents and this situation caused a loss to pharmaceutical industries.

1.2 Scope of study

The study covers the scope of microbiology involving actinoycetes isolated from marine sediment. Besides, it also includes the study of natural products produced from the isolated actinomycetes.

1.3 Premises for research

The isolation, characterization and identification of the isolates were carried out at CR1 Lab in Centre for Chemical Biology (CCB) which is a collaboration research lab. Bioassays and all the screening process using *C. elegans* were carried out at Assay Department in the Institute of Pharmaceutical and Nutraceutical Malaysia (IPharm). All the chemical works were performed at the School of Chemical Sciences, USM.

1.4 Objectives

The objectives of the study were:

i. To isolate actinomycetes from marine sediments

ii. To determine extracts with potential anti infective activity towards infection of *P. aeruginosa* PA14 in *C. elegans*

iii. To isolate and identify possible compounds with anti-infective activity by preparative TLC method and GC-MS analysis

CHAPTER 2

LITERATURE REVIEW

2.1 *Pseudomonas aeruginosa*: the common pathogen

P. aeruginosa is a Gram-negative rod shaped bacteria with the ability to anchor, colonize and spread infection to the host. Pseudomonal infection is easily detected with the presence of blue-green colourization during culture. The colourization was first observed by Sêdillot in 1850 on a surgical wound dressing (Lister *et al.*, 2009). *P. aeruginosa* can be found in almost everywhere because it is able to tolerate variety of physical conditions and able to survive with minimal nutritional requirements. Human atmosphere such as home humidifiers, swimming pools, whirlpools, hot tubs, soil and rhizosphere, vegetables and contact lens solution are among examples of this organism's reservoir (Harris *et al.*, 1984).

In the hospital, *P. aeruginosa* can be isolated from sinks, mops, respiratory therapy equipment and physiotherapy and hydrotherapy pools (Lister *et al.*, 2009). Usually acquired from hospital or health care facility, *P. aeruginosa* infection was one of the most common pathogen involved in mostly reported nosocomial infection (Diekema *et al.*, 1999) affecting almost 2 million patients and 9,000 death each year. As nosocomial infections occur during hospital stay, it caused prolonged stay, disability, and economic burden to the patients. Frequently prevalent infections are catheter-associated urinary tract infection, surgical site infections and ventilator-associated pneumonia (Khan *et al.*, 2017). Urinary tract infection is on the top list of nosocomial

infection with about 80% of infections are associated with the use of an indwelling bladder catheter (Mulcahy *et al.,* 2014). Urinary infections are associated with less morbidity than other nosocomial infections, but can occasionally lead to bacteraemia and death (Mayon-White *et al.,* 1988). Infection rates are higher among patients with increased susceptibility because of old age, underlying disease, or chemotherapy (Rafiee *et al.,* 2016). Nosocomial surgical site infections are also frequently reported. The incidence varies from 0.5 to 15% depending on the type of operation and underlying patient status. The infection usually acquired during the operation itself, either from outside sources such as air, surgeons and medical appliances or from the inside of the patients such as the flora of the patient's skin (Guggenbichler *et al.,* 2011). The ventilator associated pneumonia mostly takes place in the intensive care unit with reported incidence of 9% to 70% (Vincent, 2003). Approximately 8 to 28% of patients receiving prolonged mechanical ventilation (more than 48 hours) will develop ventilator-associated pneumonia (Cook and Kollef, 1998).

Despite all, *P. aeruginosa* is one of the common microbial florafound in human. About 2% of them colonize the skin, 6.6% colonize the throat, 3.3% colonize the nasal mucosa and 2.6 to 24% are found in fecal samples (Morrison and Wenzel, 1984). But, among patients who have records with trauma or a cleft in mucosal or cutaneous barriers by mechanical ventilation, tracheostomy, catheters, surgery, or severe burns, the colonization can exceed to 50% compared to a healthy human (Blanc *et al*., 1998; Erol *et al.*, 2004; Valles *et al.,* 2004). Disturbance of the normal microbial flora due to improper antimicrobial therapy event and patients with defective immune system have higher risks for *P. aeruginosa* infection (Morrison and Wenzel, 1984).

Many virulence factors such as adhesins, proteases, phenazines, type III secretion system exotoxins (T3SS) (Pereira *et al.,* 2014), exotoxin A, exoenzyme S and hemolysins (Delden and Iglewski, 1998) are secreted by a mechanism involving cell to cell signaling systems of *P. aeruginosa* that allow the bacteria to produce these factors in a coordinated manner upon cell density dependency. In the initial stage of infection, adhesins, such as lectins and motility features allow the bacteria to adhere to the host cells (Strateva and Mitov, 2011), while proteases degrade elastin which represents 28% of the lung tissue (Okumura *et al*., 2008). At the same time phenazines impair mitochondrial activity and the production of neutrophiles and macrophages. It also increases intracellular oxidative stress (Bradbury *et al*., 2010). On the other hand, T3SS exotoxin stimulates eukaryotic cells apoptosis and spreading of disease from the lung in pneumonia infection (Hauser, 2009). Exotoxin A causes clinical infections by catalyzing ADP-ribosylation and inactivation of elongation factor 2, causing inhibition of protein biosynthesis and later, cell death (Wick *et al*., 1990). It causes local tissue damage and bacterial infection (Woods and Iglewski, 1983). Another exotoxin known as exoenzyme S which functions like exoenzyme A, an ADP-ribosyl transferase, preferentially favoured GTP-binding proteins such as Ras (Iglewski *et al*., 1978). It leads to tissue destruction in lung infection and is responsible for bacterial invasion. Rhamnolipid and phospholipase C are two hemolysins produced by *P. aeruginosa* that work together to break down lecithin and lipids. Rhamnolipid has a structure like detergent, which solubilizes the phospholipids of lung surfactant, allowing easier access of phospholipase C to cleave the structure of lung surfactant leading to chronic and acute *P. aeruginosa* lung infection (Delden and Iglewski, 1998).

Despite all the productions and secretions, the virulence factors are controlled by quorum sensing (QS) mechanism. In 2003, Smith and Iglewski proposed a model of *P. aeruginosa* quorum sensing mechanism. The model showed that during infection, quorum sensing molecule 30-C12-HSL is produced in high concentration thus stimulating host cell to secrete multiple inflammatory mediators. The induction of interleukin, IL-8 and other mediators trigger the migration of many different cell types to the site of infection. However, as *P. aeruginosa* are protected in biofilm, immune cell such as neutrophils are unable to clear the infection and ultimately result in tissue destruction due to *P. aeruginosa* infection causing it hard to be treated. Many drugs have been invented to treat the infection of *P. aeruginosa*. Along with the developments of antibiotics, bacterial pathogens are also evolving, adapting to the antibiotic used and become resistant to it.

2.2 Antibiotic resistance the global problem

Antibiotic resistance is the problem faced globally. Pathogens that are treated with particular antibiotic sometimes are not fully cleared. Few cells that carried resistant gene will remain and cause antibiotic resistance. They performed mechanisms such as efflux pumping that channel out antibiotics from the cell, making them more difficult to be treated and we are running out of treatment option. Besides, bacteria are able to pick up resistance gene from other bacteria by using three main strategies. First, they obtain a whole plasmid coded with one or more resistance gene from a donor cell. Second, virus also plays a main role by transferring genes from one bacterium to another through

injection. The injected genes that are stably incorporated will remain in the new bacterium. And third, they scavenge gene bearing bacteria and swipe it into their environment (Levy, 1998). Figure 2.1 shows a model of bacterial cell resistance mechanism. There are three pathways (a, b and c) involved in the mechanism. Firstly, a) the genes that are coded for efflux pump play the role to channel out antibiotic out of the cell. Second, b) the genes are coded for enzyme to degrade antibiotics and finally, c) the genes that help in the chemical alteration and inactivation of the drug are delivered to destroy the pathogen's cell.

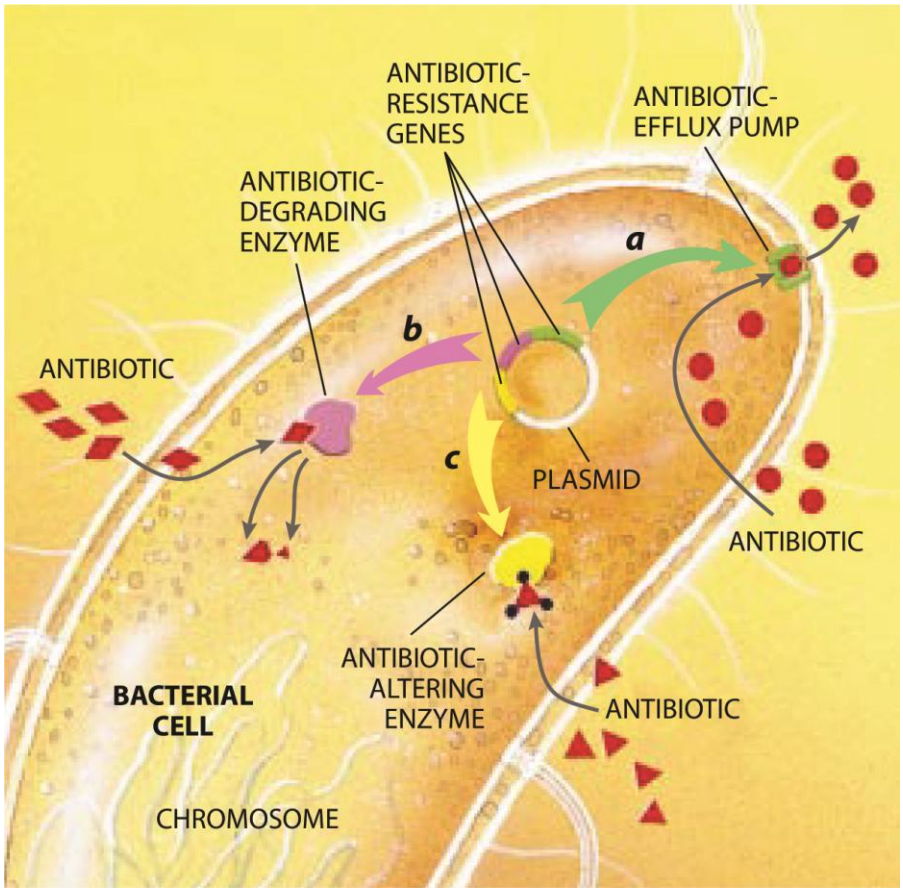

Figure 2.1: Antibiotic resistance mechanism in bacteria (Levy, 1998)

A national surveillance by using the Intensive Care Unit Surveillance Study database from ICU patients in USA showed that multidrug resistance of *P. aeruginosa* increased significantly, from 4% in 1993 to 14% in 2002 (Obritsch *et al.*, 2004). For comparison, another ICU surveillance study evaluated from 1997 to 2002 and reported an increase in case report of multidrug-resistant strains from 13% to 21% (Livermore, 2002). The number increase dramatically each year with multi-resistance to drugs (resistance to three and more drugs) such as lactams and aminoglycosides.

In *Pseudomonas earuginosa,* imported resistance mechanism and chromosomally encoded mechanism are the cause or arising multi-drug resistance phenotype. Imported resistance mechanism involved acquisition of resistance genes on mobile genetic elements such as plasmids. For chromosomally encoded mechanism, the three most studied chromosomal encoded resistance gene in *P. aeruginosa* are the AmpC cephalosporinase, the OprD outer membrane porin and the multidrug efflux pump. These encoded genes gave abilities that appoint *Pseudomonas aeruginosa* one of the greatest therapeutic challenge (Lister *et al.,* 2009). AmpC is the chromosomally encoded enzyme that is found in most Gram-negative bacteria from family *Enterobacteriaceae* and also *P. aeruginosa.* This enzyme cause resistance as it is inducible by a number of β-lactams antibiotics such as cephalothin and most penicillins along with regulation of AmpR. Upon exposure to antibiotics, *ampC* is expressed, leading to hydrolization of lactone and amides (lactams) thus causing inactivation of the drug (Jacoby, 2009).

OprD is a transport protein that facilitate transportation of basic amino acids and β-lactam carbapenem, imipenem into the cells. Resistance mechanism occur with the lost of function of OprD in which antibiotic could not be transported into the cell (Osch

et al., 1999). This might happen due to deletion of *oprD* causing frameshift mutation and generation of premature termination codon (Yoneyama and Nakae, 1993). Efflux pump is also a specific transportation method into the cell. A study on the multidrug efflux pump made up of three main components: the cytoplasmic membrane associated drug-proton antiporter of the Resistance Nodulation-Division (RND) family, outer membrane factor (OMF) which is a channel-forming protein and the periplasmic membrane fusion protein (MFP) was carried out. There are about four RND-MFP-OMF type multidrug efflux pump of *P. aeruginosa* that have been described which are MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM (Poole, 2001). Generally, these efflux pumps lead to resistance as they exported out the compounds from inside the cell to outside of the cell (Webber and Piddock, 2003). Therefore, drugs transported into the cell are also exported out.

Antibiotic resistance and antibiotic limitation have been an alarming problem that urged researchers to find more efforts in solving it. It initiatively make way to adoption of different new strategies such as anti-infective agents which not only combat the pathogen after infection but take care of the infection at the early stage.

2.3 Anti-infective agents

Drugs.com website describe anti-infective agent as an agent with the ability to react upon infection. It may act by suppressing transmission of infection or by directly killing the infectious agent. Antibiotics, antifungals, antibacterials, antivirals and antiprotozoans are generally referred to as anti-infective agents. From other perspective,

anti-infective agent selectively acts by modulating the immunity of the host or by disrupting the virulence-mediated pathways without affecting microbial cell viability (Kong *et al*., 2014b). Therefore, in order to avoid global problems of antibiotic resistance, initiative of using anti-infective agent should be taken into account.

The awareness of antibiotic resistance problem had risen since early 1988 when resistance towards vancomycin was also reported. Shimmel and co-workers (1998) firstly suggested synthase as the key to combat antibiotic resistance. They suggested the use of aminoacyl tRNA synthase, a universal enzyme as target for new drugs discovery that able to inhibit pathogen infection but not the host cells counterpart. They also demonstrate efficacy of the synthase inhibitor by using animal model in vivo.

One of the well-known anti-infective compounds is the anti-microbial peptide (AMP), the small molecular weight positively charged proteins with both hydrophobic and hydrophilic side. This characteristic enables it to be soluble in both aqueous and lipid membrane environment. It exhibits a broad spectrum antimicrobial activity against viruses, bacteria and fungi (Izadpanah and Gallo, 2005). This AMP plays a crucial role as effectors of innate immunity in most living organisms on skin and mucosal surfaces since 2.6 billion years ago (Gordon *et al.*, 2005). Most AMPs are produced by the cells itself but the production of AMPs can be stimulated through vaccination or upon infection of specific cells by pathogen (Bahar and Ren, 2013). AMP have been reported to exhibit anti-infective properties in *Staphylococcus epidermidis* with no cytotoxic effect to the mammalian cells. Natural AMP showed that AMP target the lipid bilayer membrane of pathogen while synthetic AMP designed and enhanced to target diverse range of target even at a very low concentration (Agarwal *et al.*, 2016).

Instead of small peptide compounds, there are groups that go for natural product screening for anti-infective option and more efforts in finding novel anti-infective agents were carried out because anti-infective screening of natural products from medicinal plant and microorganisms showed potential source of anti-infective agents. Medicinal plant extract from Mauritians flora exhibit anti-infective properties towards tested pathogens including *P. aeruginosa* with 57.1% susceptibility to the tested extract and 100% susceptibility towards *Bacillus subtilis* (Rangasamy *et al.,* 2007). Besides that, a whole organism infection model employed in anti-infective screening towards *P. aeruginosa* involving host treated with seed extract of *Swietenia macrophylla* from Malaysian environment also demonstrated a positive result with 59.5% of the host survived the infection of *P. aeruginosa* PA14 upon treatment of the mentioned extract (Dharmalingam *et al.,* 2012). In comparison, Rudrappa and Bais (2008) also demonstrated anti-infective screening of curcumin extract that save the host towards infection of *P. aeruginosa* PA01 by reduction of virulence factor expression of the pathogen.

Instead of natural product from plant, products from microorganisms were also screened for potential anti-infective activities. Marine bacteria *Verrucosispora* AB-18-032 showed good inhibitory activity against MRSA (methicillin resistant *Staphylococcus aureus*) and VRSA (vancomycin resistant *S. aureus*) with discovery of abyssomicin C (Rahman *et al*., 2010). On the other hand, *Streptomyces* sp. isolated from marine sponge reported to exhibit anti-infective properties and also led to a very first discovery of valinomycin from the marine source (Pimentel-Elardo *et al*., 2010).

2.4 *Caenorhabditis elegans* as host infection model

2.4.1 *C. elegans* biology

C. elegans host model system has been introduced by Sydney Brenner in 1965. C. *elegans* is a nematode species that can be found all over the world. They inhabit leaf litter and are saprophytic (Hope, 1999). It has a simple anatomy which plays a crucial role in the selection as a model organism (Brenner, 1974). It has been widely used as it is very easy to be cultivated in the laboratory and it has a short life-cycle of about 3 days of cultivation (Riddle *et al.,* 1997). *C. elegans* goes through four larval stage (L1- L4), from hatching to adult. Incident of food depletion and pheromone influence at L2 stage will drive the larvae into an alternative juvenile stage called the "dauer" larvae which can live up to four months without food source (Golden and Riddle, 1982). Figure 2.2 shows life cycle of *C. elegans* from eggs to adult and probability of juvenile arrested 'dauer' stage.

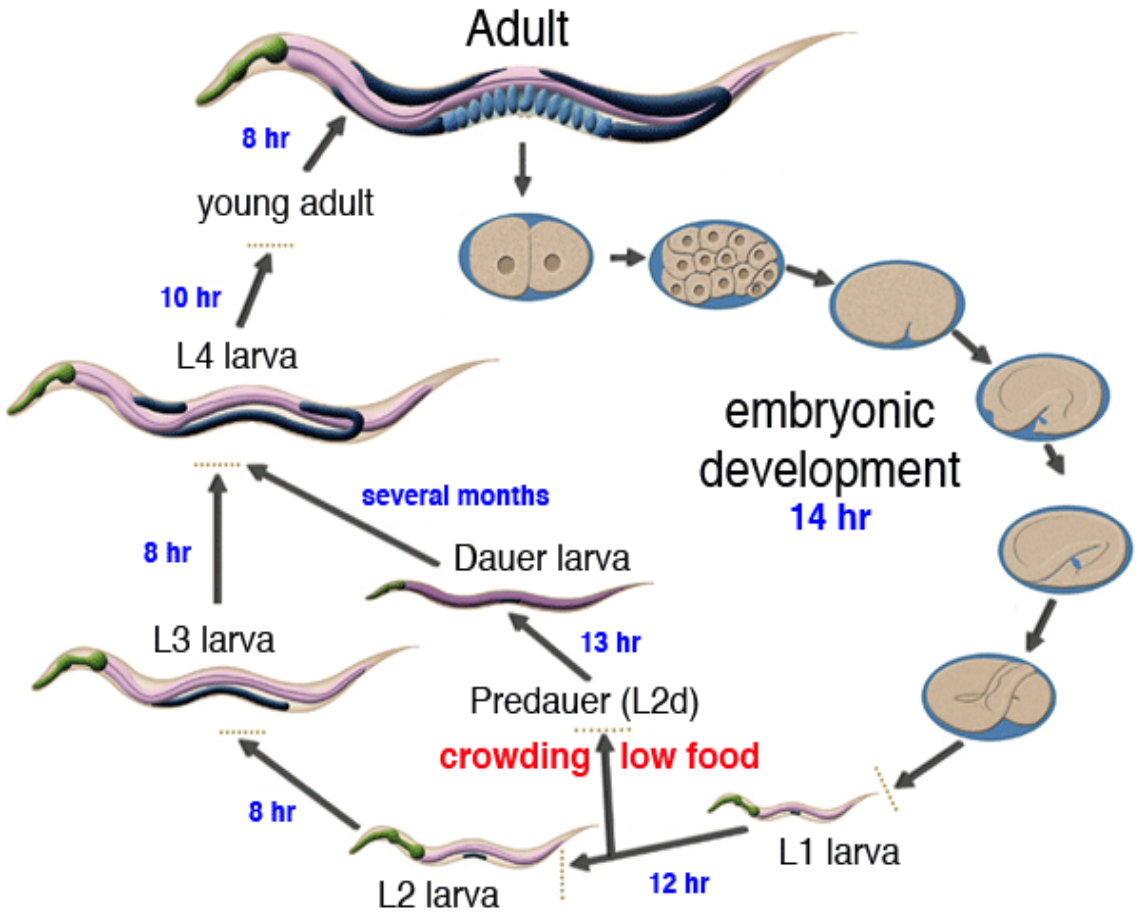

Figure 2.2: Life cycle of *C. elegans*

Image source: http://www.wormatlas.org/ver1/handbook/anatomyintro/anatomyintro.htm (accessed on November 18, 2015).

Adult worm is about 1.5 mm in length and produced about 300-350 progeny per worm. The use of the worms as an potential host is comparable to the T4 phage by the number of progeny produced while the breeding method are comparable to the one in the plant (Riddle *et al.*, 1997). *C. elegans* complements both *in vitro* and *in vivo* mammalian models in toxicology due to its success in genetic manipulability, invariant and fully described developmental program and well-characterized genome (Leung *et*

al., 2008). The invariant number of somatic cells in *C. elegans* allows a full track of every significant cell developmental changes such as the neuronal, hypodermal, muscle and digestive cells. The changes can be tracked starting from egg-fertilization until adult stage so that the fate of every cell is known (Sulston and Horvitz, 1977). *C. elegans* protein-coding genes presumed that about 38% of *C. elegans* gene have clear orthologs in human genome (Shaye and Greenwald, 2011) and 60-80% of human genes have ortholog in *C. elegans* genome (Kaletta and Hengartner, 2006). Culetto and Sattelle (2000) reported that about 40% of known genes that are related to human disease have orthologs in *C. elegans* genome. Therefore, it urges adoption of different strategies in order to seek new anti-infective therapeutic agents including practices by using the whole animal infection model such as *Caenorhabditis elegans* (Kirienko *et al*., 2013; Moy *et al*., 2006).

2.4.2 *C. elegans* host-pathogen interaction

Mammalian innate immunity is far too complicate to be directly studied. Therefore, *C. elegans* is listed among the best candidate for host-pathogen interaction for innate immunity study. Earlier, Drosophila and mouse was also used to study effects of anti-microbial peptide on innate immunity of the host but then it revealed the complicated functional conservation of Toll signalling between insect and human (Poltorak *et al.,* 1998). Therefore, *C. elegans* was used to get a clearer idea of the mammalian innate immunity studies. *C. elegans* also provide a genetically tractable mechanism of host defense (Kim, 2008).

Besides, the ability of *C. elegans* that allows various pathogens to establish an effective infection allows assessment of bacterial pathogenesis with any pathogen of interest. Three modes of infections that are the intestinal infection, cuticle infection and infection of tail region that cause swelling response are suggested to elicit immune response of the host (Kim, 2008). This mode of infection allows various killing mechanism to be studied. Kong and co-workers (2014b), studied interaction between *C. elegans* and *Staphylococcus aureus* upon infection while Rudrappa and Bais (2008) used *Pseudomonas aeruginosa* PA01 as the pathogen. More pathogens were actually used such as *Burkholderia pseudomallei* (Eng and Nathan, 2015), *Microbacterium nematophilum* (Hodgkin *et al.,* 2000) and *Streptococcus pneumonia* (Jansen *et al*., 2002).

On the other hand, there are signalling pathways and genes that are homolog to humans system. The conserved pathway of immune signaling in *C. elegans* PMK-1 p38 mitogen-activated protein kinase (MAPK) pathway is in relative position with mammalian apoptosis signal-regulating kinase 1(ASK1)-MAPK kinase 3/6 (MKK3/6)-p38 MAPK pathway involved in innate immunity (Aballay *et al*., 2003). Another gene homolog to human is the *tol-1* that homolog to Toll-like receptor (TLR) (Pujol *et al.*, 2001). Next gene involved is *tir-1* that ortholog to human TIR (Transport Inhibitor Response) domain protein MyD88-5 whose responsible in primary responses in human (Mink *et al.,* 2001). Therefore, TIR-1 is related to the function of ASK1-dependent PMK-1 p38 MAPK pathway during defense towards pathogen (Liberati *et al*., 2004). In AWC chemosensory neuron, TIR-1 was also reported to play role in neuronal

development as it is responsible in ASK1-dependent activation of a MAPK distinct to PMK-1 (Chuang and Bargmann, 2005).

Besides, the well-studied genetic of *C. elegans* has led to the study of gene expression of host upon pathogen infection. It suggested that infection of pathogen induced production of common classes of genes associated with lysozyme and C-type lectin domain-containing protein as defense mechanism of the host towards infection (Kim, 2008). Further microarray based study upon these genes were also carried out and a comparative analysis of these genes with genes regulated by the PMK-1 showed that about 15% of PMK-1-regulated genes are also induced by *P. aeruginosa.* This further proved that the PMK-1 pathway is related to *C. elegans* innate immune response upon pathogen infection (Troemel *et al*., 2006).

Moreover, the study of host-pathogen relation in *C. elegans* does not only involve the tissue on site of infection. This is because *C. elegans* are able to exhibit behavioral changes upon infection. Besides, physiological processes such as reproduction and growth are also related to defense mechanism towards pathogen (Kim, 2008).

2.4.3 *C. elegans* in drug discovery and screening

Among the earliest *C. elegans* research has started since 1900 when Maupas successfully identified *C. elegans*. Then in 1948, Dougherty and Calhoun proposed to use *C. elegans* in their genetic research. After that, Brenner took a first step in 1963 by obtaining *C. elegans* sample from Dougherty and started his molecular-genetic work in

collaboration with Sulston until the genetic sequence was completed in the year of 1997 (Riddle *et al.,* 1997). Since then, *C. elegans* has been a famous manipulation tool and extensively used in drugs discovery, screening and evaluation as compared to the traditional cell drug screening method.

As compared to the traditional cell drug screening, the whole animal infection model of *C. elegans* allows an early and direct assessment of *in vivo* drug efficacy. At the same time, compounds that cause toxicity to the host and compounds with poor pharmacokinetic properties are eliminated. Besides, it allows identification of potential hits that selectively disrupt virulence pathways established by pathogens (Moy *et al*., 2009). *C. elegans* infection model give opportunity to simultaneously identify compounds that not only target the bacterial virulence factor but also modulating the host defence (Kong *et al*., 2014b). Therefore, based on the concept, the selectivity of anti-infective in which only non-essential genes are targeted for disruption feasibly impose a lower probability of resistance development (Mellbye and Schuster, 2011) and at the same time preserve cell viability of the host.

Note that, *C. elegans* was not only used for anti-infective screening but a lot more was done. The first method of drug screening using *C. elegans* host infection model was carried out by Brenner in 1974 by incorporating the drug in the agar during screening. After that, this model organism was used in more drug screening practices such as in anti-aging research in 1977 by Klass who stated that this model allowed a consistent life-span to be measured with manipulation of food source and incubation temperature (Tissenbaum, 2015). This agar screening model was also used to model mammalian bacterial pathogenesis the *P. aeruginosa* PA14 (Tan *et al.,* 1999). In 2012,

Dharmalingam and co-workers used the same *C. elegans* agar screening method to screen *Swietenia macrophylla* seed extract efficacy towards infection of *P. aeruginosa* PA14. However, this agar based method consumed large amount of compound and laborious. Therefore, it was improved to High Throughput Screening (HTS) with higher resolution and efficiency.

The first large scale of high throughput screening was reported on 2006 carried out by Kwok and colleagues. They use fully automated worm transfer using a Complex Object Parametric Analyzer and Sorter (COPAS™ BIOSORT, Union Biometrica) and semi-automated image acquisition to screen 14,100 small molecules and identified 308 bio-active compounds (Kwok *et al.,* 2006). Moy and co-workers (2006) are among the earlier group to perform liquid-based screening assay using *C. elegans.* They successfully screened 1,136 natural product extract and 6,000 synthetic compounds towards infection of *Enterococcus fecalis* and found that out of the number, 16 compounds and 6 extracts improved survival of host upon pathogen infection. Next, anti-fungal of 1,266 compounds with known pharmaceutical activities were screened towards *Candida albican-C. elegans* infection model through HTS and 15 of the compounds showed positive result (Breger *et al.,* 2007). In 2011, Zhou and co-workers screened 1,300 extracts for accessing bio-activity of the extracts towards infection of *P. aeruginsa* in *C. elegans* and they discovered 36 extracts that improve survival of the host upon infection.

As discussed above, it proved that *C. elegans* has been extensively used as the tool for drug discovery for about four decades and many of its innate immunity and microbial pathogenesis are well studied by using this model (Ewbank and Zugasti,

2011). Therefore, this work has been carried out to study the main component produced by the actinomycetes that act as immune modulator of the host upon infection of *P. aeruginosa*.

2.5 Introduction of actinomycetes

Bacteria which belong to the order Actinomycetales were informally called aerobic actinomycetes. Microorganisms of this order were originally classified as fungus as they possess the same characteristic, the true aerial hyphae. Later on, further studies were carried out on their cell wall component particularly the cell envelope lipid and peptidoglycan compositions. They are now classified as the true aerobic bacteria (McNeil and Brown, 1994). Actinomycetes are typically Gram-positive filamentous free living bacteria that are saprophytic. They grow slower than common bacteria which take up to three weeks to be seen clearly on the agar plate particularly on media rich in protein. At the early stage, actinomycetes colonies are very hard to be differentiated among common bacteria colonies. But longer incubation time make it easier to be seen as a chalky deposit which often appear around the colony. Sometimes it forms a concentric ring around it (Haines, 1931).

Actinomycetes colonies are usually found in the form of white powdery, leathery, pinpoint and creamy (Gebreyohannes *et al.*, 2013; Valli *et al.*, 2012). Most actinomycetes produce coloured pigment in their colonies as their secondary metabolites (Demain, 1998). Actinomycetes have a special recognition based on their smell of earth which actually comes from the substance secreted by actinomycetes called geosmin.

This event caused the smell of soil during growth of actinomycetes (Gerber and Lechevalier, 1965).

Actinomycetes also produce dry spores like most fungi (Kalakoutski and Agre, 1976) which are airborne contaminants in most working places such as agriculture land and waste composting site (Lacey, 1989). The variety and abundance of actinomycetes in any specific environment is mostly depends on the type of cultivation, geographical location, soil and organic matter among other factors (Arifuzzaman *et al.,* 2010). Not only presented in the soil, it can be found in almost all kinds of environments such as freshwater, air and marine environments (Mohseni *et al*., 2013). Experts estimate that the biological diversity is higher in some marine ecosystems such as the deep sea floor and coral reefs compared to that in the tropical rainforests. In the sea, actinomycetes also live in association with other microorganisms and they mediate the degradation and cycle the organic matter (Jensen *et al.,* 2005a; Lam, 2006).

Actinomycetes are not only found in marine and terrestrial environment but also can be found in an extreme environment with very low temperature such as Antarctica in which 98% of the areas are covered with thick sheets of ice. Despite the extreme low temperature, rainfall and water is also scarce (Prabahar *et al*., 2004). Nedialkova and Naidenova (2004) successfully isolated a total of 40 actinomycetes strains from Antarctica soil sample while Fink and co-workers (1971) have isolated actinomycetes from genus *Thermoactinomycetes* from heating system of office buildings proving that some actinomycetes also live in thermophilic environment.

Actinomycetes are rarely related to clinical practises and diseases but some of them may cause serious infection in human and animal (McNeil and Brown, 1994). Actinomycosis is one of the slow progressive infections caused by oral and gastrointestinal communal, *Actinomycetes israelii*. It is one of the chronic granulomatous disease (CGD) that commonly manifests as pulmonary, cervicofacial, or abdominal disease (Jahromi and Dootskam, 2011). Actinomycetes form a compelling component of the commensal microflora of the gastrointestinal, oral and female genital tracts and they generally have low pathogenicity. They may infect a body via breached mucosa, governing bacteremia and systemic infections in normal individuals and immuno-compromised patients (Clarridge and Zhang, 2002). Medication prescription of actinomycosis is usually easy in immunocompetent individuals by giving long-term and high-dose intravenous penicillin. It is more difficult in those with chronic granulomatous disease (CGD) patient because of delayed diagnosis and the increased risk of chronic invasive or incapacitates disease (Reichenbach *et al*., 2009).

As it may cause diseases, actinomycetes also may produce variety of drugs as they are also known as a major source of antibiotics. Actinomycetes have been discovered to be a supply reservoir of medicinal antibiotics and remarkably important to agricultural industries, pharmaceutical industries and scientists (Kumar *et al.,* 2010). Majority of the presently used antibiotics including streptomycin, erythromycin, gentamycin and rifamycin are all isolated from soil actinomycetes (Jeffrey, 2008). *Streptomyces* and *Micromonospora* are two major groups of soil actinomycetes that serve as important sources of antibiotics. It was discovered that genus *Streptomyces* alone contribute about 80% of the total medical antibiotic products; while

genus *Micromonospora* follows with less than one tenth as much as *Streptomyces* (Arifuzzaman *et al*., 2010). It has been proven by previous experimental analysis that the secondary metabolites isolated from soil actinomycetes are potential suppressor of numerous plant pathogens (Jeffrey, 2008). In agriculture field, *Erwina amylovora*; a bacterium that causes fireblight to apples and *Agrobacterium tumefaciens*; the causative pathogen of crown gall disease in plants has been proved to be inhibited by actinomycetes isolated from the farming soil (Oskay *et al*., 2004). There are 21 potential actinomycetes isolated by Valli and co-workers (2012) from marine environment that are reported to be the promising secondary metabolites producer against at least one tested organism. Studies have proven that actinomycete*s* isolated from the marine environment are capable of producing novel secondary metabolites and have adapted to life in the sea. *Streptomyces* can produce a variety of secondary active metabolites and medicinal antibiotic (Thenmozhi and Krishnan, 2011). Marine actinomycetes have different characteristics from those of terrestrial group because of marine environmental conditions that are extremely different from terrestrial. So, it gives a run down that they might produce different types of bioactive compounds (Imada *et al.*, 2007). Actinomycetes that grow in the ocean are well adapted to the marine environment especially toleration to high Na^+ and Cl^- ion concentration and high hydrostatic pressure. The adaptation made it an interesting organism to be studied as it may produce novel therapeutic agents (Subramani and Aalbersberg, 2012).

Nedialkova and Naidenova (2004) have successfully isolated a total of 40 actinomycetes strains from Antarctica soil sample. All of the isolates were screened for antimicrobial bio-activity against seven Gram-positive and Gram-negative bacteria and